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(54) Title: CONJUGATES OF SUPEROXIDE DISMUTASE (57) Abstract A water-soluble substantially non-immunogenic conjugate of superoxide dismutase is prepared by coupling to a polyalkylene glycol which is polyethylene glycol or polyethylene-polypropylene glycol copolymer, wherein said glycol has an average molecular weight of 40,000-1,000,000 and is terminally unsubstituted or substituted by C ₁₋₄ -alkyl or C ₁₋₄ -alkoxy groups.		

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CONJUGATES OF SUPEROXIDE DISMUTASE

The present invention is directed to superoxide dismutase (SOD) conjugates or adducts in which at least a portion of the SOD amino, carboxyl, or sulfhydryl groups are coupled to a polyalkylene glycol (PAG), such as a polyethylene glycol (PEG) or a polyethylene-polypropylene glycol copolymer, wherein the PAG has a molecular weight greater than 20,000 and desirably an average molecular weight of 40,000 to 1,000,000 daltons. Unless otherwise indicated, molecular weights for PAG are those determined by high performance, size exclusion liquid chromatography (HPLC) using PEG as standard. Preferred is a PAG with an average molecular weight greater than 40,000 and no greater than 200,000 and especially preferred is an average molecular weight for the PAG of 50,000-150,000. Such PAG can be linear or branched and be unsubstituted or substituted by C₁₋₄-alkyl or C₁₋₄-alkoxy groups. Of special value to prevent linkage to two SOD molecules are PAG molecules in which one terminal group is a C₁₋₄-alkyl ether group such as an isopropoxy group.

BACKGROUND OF THE INVENTION

Previous workers have utilized PEG or methoxy-PEG of low molecular weight, and typically about 5,000 and less, attached to superoxide dismutase (SOD) and other proteins to obtain adducts demonstrating varying degrees of (a) increased serum persistence and (b) reduced immunogenicity. However, the extent of modification of protein groups with low molecular weight PEG or methoxy-PEG required to adequately attain both objectives (a) as well as (b), often leads to substantial losses in enzyme activity or biological activity.

DETAILS OF THE INVENTION

Superoxide dismutase (SOD) is an intracellular enzyme responsible for catalyzing the conversion of the superoxide

radical to oxygen and hydrogen peroxide. It is an object of the invention to provide a product which is more persistent than the native SOD protein in vivo and to delay inactivation by the kidneys. It is especially important
5 that the product retain enzymatic activity for a prolonged time while exhibiting a low level of immunogenicity.

It is also a special object of this invention to provide a conjugate with anti-inflammatory activity, as shown, e.g., in the conventional carrageenan-induced paw edema
10 test. This activity makes the product especially promising for treatment of rheumatoid conditions.

The novel concept embodied in the present invention utilizes high molecular weight PAG strands, greater than 20,000. The PAG used in the present invention can be any
15 water soluble polymer of alkylene oxide, for example, poly(ethylene oxide) or certain copolymers of ethylene oxide and propylene oxide. The PAG can be linear or branched and can be substituted at one or more hydroxyl positions with a C₁₋₄-alkoxy group or other chemical group.
20 However, the molecular weight of the PAG polymer used for the preparation of the conjugate in the present invention is greater than 20,000 and preferably in the 40,000 to 200,000 MW range. Use of polymers larger than 200,000 is also possible but they are not preferred due to their
25 higher viscosity and susceptibility to cleavage by shearing.

The PAG-SOD adducts of the present invention have molecular weights (relative to PEG standards of known molecular weight) ranging from about 85,000 to about
30 2,000,000 daltons, and preferably about 90,000 to 1,000,000 daltons. Furthermore, the PAG-SOD adducts and other PAG-adducts of the present invention usually retain most of the enzymatic activity of the native protein. (It is to be noted that molecular weights herein are based on PEG

standards of known molecular weight. For the purpose of HPLC calibration, the protein equivalent molecular weights, i.e., MW based on protein standards of known MW appear to be approximately 5 to 8 times larger).

5 The conjugates of the present invention are an improvement over previous products in that by attaching fewer PAG strands, less chemical modification of the active parent molecule results, so that more of the original character of the parent molecule is preserved. Thus, by
10 using fewer strands of high molecular weight PAG, as in the present invention, the adducts retain most, if not substantially all of the activity of the parent molecule, while also demonstrating increased persistence in the bloodstream. Another advantage of the adducts of the
15 present invention is that by using high molecular weight PAG, larger adducts can be made with the same degree of modification attained by other workers. Furthermore, in some applications, larger PAG adducts are clearly advantageous. For example, PEG-SOD adducts of this
20 invention, which were prepared using PEG-strands in the 40,000-130,000 MW range by the methods of this invention and which illustrate the principle of this invention, had serum half-lives in mice of about 36 hours and greater, longer than the PEG-SOD adducts described by previous
25 workers.

The PAG-SOD adducts preferably contain from 1 to 10 chains of attached PAG per protein molecule, and more preferably 2 to 8 and especially 2 to 6 chains of PAG per molecule. The number of chains needed to achieve
30 satisfactory serum persistence decreases when longer chains are used.

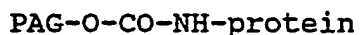
The SOD preparations of this invention are typically the mammalian copper-and zinc-containing superoxide dismutase forms of the enzyme derived from bovine, other animal

(e.g., sheep, horse, pork, dog, rabbit, chicken) or human cells. Also available are SOD preparations containing other metal ions such as iron or manganese. Also useful is the enzyme with congeneric structures derived from
5 microbial cultures in which such structures have been cloned and expressed. The SOD may also be non-identical to the naturally occurring proteins as a result of infidelity of translation in such microbial cultures, since the products of this invention have reduced immunogenicity.

10 It has also been found that when the SOD preparation contains traces of non-SOD proteins which would otherwise make such preparation immunologically unsafe for repeated parenteral administration, coupling such SOD by the methods of this invention can render the product reasonably useful,
15 since the impurity proteins are rendered substantially less immunogenic.

In the coupling process, a number of conventional reactions can be used.

A preferred reaction proceeds by way of formation of a
20 reactive carbonate half ester, PAG-O-CO-X, wherein X is a good leaving group, using reagents such as carbonyl diimidazole, p-nitrophenyl chloroformate or bis-N-succinimidyl carbonate. The activated PAG, PAG-O-CO-X, is then reacted with the protein under conditions which do not
25 destroy its enzymatic activity, leading predominantly to urethane linkages



attached through protein amino groups such as the epsilon-NH₂ group of lysine.

30 For example, carbonyl diimidazole, can be reacted with the terminal hydroxyl groups of the PAG. The reaction mixture is quenched in aqueous solution at neutral pH and the activated PAG (polyalkylene glycol-carbonyl-imidazole)

is isolated by dialysis and/or size exclusion chromatography.

Reaction of the $\text{PAG-O-CO-N} \begin{array}{c} \diagup \text{CH} = \text{N} \\ \diagdown \text{CH} = \text{CH} \end{array}$ with SOD is carried

5 out in solution, with an excess of activated PAG.

In a variant of this reaction, a solution of SOD and activated PAG is freeze-dried. The coupled products are conveniently isolated by size exclusion chromatography. Other purification processes can be employed including ion
10 exchange chromatography.

In an alternate coupling reaction, the polyalkylene glycol is dissolved in an inert organic solvent, the reaction mixture is rendered weakly alkaline and reacted with cyanuric chloride.

15 The unreacted cyanuric chloride is removed by precipitating the PAG with petroleum ether. The residual solvent is evaporated to yield a 2-PAG-6-chloro-1,3,5-triazine. The resulting activated polymers are then reacted with SOD in a suitable buffer, e.g., a borate
20 solution. The unreacted activated PAG is removed and the product isolated by chromatography. There is thus obtained a 4-hydroxy-1,3,5-triazine to which there is attached at the 2-position the polyalkylene glycol group PAG-O- while at the 6-position there is attachment to the epsilon-amino
25 group of a reactive lysine group of SOD.

One or more hydroxy groups of PAG-OH can also be converted to a carboxyl group, e.g., by reaction with succinic anhydride or with ethyl bromoacetate and alkali, or by oxidation of the terminal $-\text{OCH}_2\text{CH}_2\text{OH}$ with alkaline
30 permanganate to form the PAG acetic acid ethers, $\text{PAG-O-CH}_2\text{-COOH}$. The carboxyl groups are then activated by methods commonly known to be useful for protein modification, e.g., formation of the N-hydroxy succinimide ester by reaction with a carbodiimide and N-hydroxysuccinimide, or formation

of the acyl azide by nitrosation of the acyl hydrazide. The activated PAG is then reacted with the protein under conditions which do not destroy the enzymatic activity of the protein, leading predominantly to amide linkages (PAG--
5 -C(=O)NH-protein) via protein amino groups (such as amino terminal NH₂ and lysine epsilon amino groups).

A terminal PAG hydroxyl group can also be converted to an amino group, e.g., by reaction first with thionyl bromide to form PAG-Br, followed by aminolysis with excess ammonia
10 to form PAG-NH₂. The amino-PAG can then be coupled through amide bonds directly to the protein carboxyl groups by use of reagents such as water-soluble carbodiimide or Woodward's Reagent K. Alternatively, the amino function can be converted to a carboxylic acid function, e.g., by
15 reaction with succinic anhydride, which is then activated and reacted with the protein in the manner described above.

The PAG terminal -CH₂OH can also be converted to an aldehyde group, -CH(=O) e.g., by oxidation with MnO₂. The aldehyde group can then be reductively alkylated onto the
20 protein via the latter's free amino groups, e.g., with cyanoborohydride, to give a linkage predominantly via secondary amine groups, forming a PAG-OCH₂CH₂NH-protein bridge.

In addition to protein amino groups, protein carboxyl and
25 sulfhydryl groups can also be used for coupling to the PAG.

As stated above, in selecting coupling reactions, those are preferred which leave only non-aromatic groups composed of carbon, oxygen, sulfur, nitrogen and hydrogen as part of the bridge linking the PAG to the protein.

30 The conjugated SOD can be isolated from the reaction solution, preferably after dialysis to remove extraneous ions, by conventional lyophilization. If desired or necessary, the conjugate can be further purified by ion

exchange chromatography, electrophoresis, and/or gel filtration.

Filtration through a micropore filter in a conventional manner into sterile vials, optionally after adjusting ionic strength, e.g., with sodium chloride and/or sodium phosphate to isotonicity, provides a sterile solution suitable for administration by injection.

The pharmaceutical compositions of this invention comprise PAG-SOD conjugates of this invention and a pharmaceutically acceptable carrier.

The pharmaceutical composition preferably is in the form of a sterile injection preparation, for example a sterile injectable aqueous solution. The solution can be formulated according to the known art using pharmaceutically acceptable carriers mentioned above. The sterile injectable preparation can also be a solution or suspension in a non-toxic parenterally acceptable diluent or solvent.

The compositions of this invention combine an effective unit dosage amount of SOD conjugate at a concentration effective to evoke the desired response when a unit dose of the compositions is administered by the route appropriate for the particular pharmaceutical carrier. For example, liquid compositions usually contain about 0.5 to 40 mg of conjugate protein per 0.25 to 10 ml, preferably about 0.5 to 5 ml, except I.V. infusion solutions, which can also be more dilute, e.g., 0.5 to 200 mg SOD conjugate protein per 50-1,000 ml, preferably 100-500 ml, of infusion solution. Tablets, capsules and suppositories usually contain 0.1 to 25 mg, preferably 1 to 10 mg, of conjugate protein per dosage unit.

The SOD conjugates of this invention, like the established product orgotein, are effective in treating a wide variety of inflammatory conditions, including those in

which synthetic anti-inflammatory agents have limited utility, e.g., because of toxic side effects upon prolonged use.

More specifically, the SOD conjugates are efficacious in ameliorating oxygen toxicity, reperfusion injury, and inflammatory conditions and mitigating the effects thereof, for instance those involving the urinary tract and the joints, in various mammals. They are useful in alleviating the symptoms of and the structural deformities associated with post-traumatic arthritis, and rheumatoid diseases, such as bursitis, tendinitis, and osteoarthritis.

The invention is further illustrated by the following examples.

EXAMPLE 1

Fifty grams of PEG (a Union Carbide product labelled Polyox^R or polyethylene oxide 100,000, consisting of isopropoxylated PEG with a labeled molecular weight of 100,000 as determined by intrinsic viscosity measurement for which HPLC gave an average value of about 50,000) was dissolved in 1 liter of dry pyridine and 22 grams of succinic anhydride was added. The mixture was stirred for 38 hours at 60°C. The solvent was removed under vacuum at less than 60°C and the residue was redissolved in 500 ml of water. The solution was washed with hexane and then the product was extracted into 1000 ml chloroform. The chloroform was removed under vacuum at 40°C and the product was dissolved in benzene. The succinylated PEG was reprecipitated twice from benzene with petroleum ether.

An aqueous solution of this succinylated PEG was size-fractionated to remove low molecular weight PEG by ultrafiltration using a Millipore^R Minitan^R apparatus equipped with a 300,000 (protein standard) molecular weight cut-off membrane. The size-fractionated product was dried under vacuum. Analysis by aqueous size exclusion HPLC

indicated that the average molecular weight of the succinylated PEG had increased from about 50,000 to about 80,000 as a result of ultrafiltration. The size-fractionated succinylated PEG thus obtained was then
5 activated with N-hydroxysuccinimide. 12 gm of the succinylated PEG (molecular weight 80,000, 0.15 mmoles) was dissolved in 120 ml of dry dimethylformamide and 300 mg. (2.6 mmoles) of N-hydroxysuccinimide was added, while stirring. After dissolution, 2.4 mmoles of
10 dicyclohexylcarbodiimide was added and the solution was stirred at 40°C for 30 minutes and then left unstirred at 24°C for five days. The mixture was then filtered through a glass fiber filter and the filtrate was evaporated to dryness under vacuum at 40°C. The residue was dissolved
15 with mild heating in 200 ml of dry toluene. The solid N-hydroxysuccinimidyl PEG was precipitated by addition of 400 ml of petroleum ether. The product was collected under vacuum on a glass fiber filter and then reprecipitated from toluene using petroleum ether, and then dried under vacuum.
20 Size-exclusion HPLC showed that the molecular weight of the product had not changed as a result of activation.

A solution containing 80 mg of bovine Cu,Zn SOD (2.46 micromoles, 4400 Units/mg., from DDI Pharmaceuticals, Inc.) in 39 ml of 0.1M potassium phosphate buffer (pH 8.0) was
25 added to 4 grams of the dry N-hydroxysuccinimidyl PEG derivative (50 micromoles) and the mixture was dissolved at 24°C. Size exclusion HPLC analysis showed that the coupling reaction was essentially complete within 1.5 hours, as was evident from the disappearance of unreacted
30 SOD and appearance of a high molecular weight UV-absorbing adduct peak with a molecular weight of about 200,000.

The free PEG which had not coupled to SOD was separated from the PEG-SOD adduct by ion-exchange chromatography. Fractions containing PEG-SOD were collected by increasing

the ionic strength of the elution buffer (pH 9) using NaCl. PEG-SOD fractions were dialyzed against water to remove buffer components and then concentrated by freeze-drying or vacuum evaporation.

5 As an example, the properties of a typical product eluted with 50 mM NaCl are described. The adduct contained 24 mg of SOD protein and 165 mg of protein-bound PEG. The protein content was determined by biuret analysis and the PEG content by HPLC, using refractive index detection (RI)
10 and correcting for the RI contribution of the protein. The average molecular weight determined for the protein-bound PEG was 72,000. This MW for the PEG released from the adduct by proteolysis, combined with data showing that the ratio of SOD protein (32,000 MW) to PEG in the adduct was
15 24 mg to 165 mg, gives a ratio of 3 strands of PEG bound per molecule of SOD. The number of PEG strands per SOD molecule obtained in this way gives a calculated adduct MW of 220,000 ($72,000 \times 3 + 32,000/8$), a result which is consistent with the MW obtained by HPLC.

20 The SOD activity of the above adduct was determined using the cytochrome-C assay of McCord and Fridovich (J. Biol. Chem. 244: 6049-6055; (1969)). The specific activity of the PEG-SOD adduct (about 4317 units/mg protein) was 98% that of the native enzyme starting material (4400
25 units/mg). The product thus still retained almost all of the native enzymatic or biological activity, while satisfying other requirements of the invention.

The PEG-SOD derivative obtained as described in this Example was compared to the highly purified, unmodified SOD
30 starting material for immunologic sensitization potential (activity to cause anaphylactic reactions) in adult female Swiss Webster mice using a sensitization/challenge test. Ten mice were immunized by 4 subcutaneous injections during 2 weeks with 0.075 mg of protein per dose and then

challenged intravenously with the same compound with 0.04 mg of protein at 21 day intervals thereafter. Whereas by the 5th intravenous challenge, in the group receiving unmodified SOD, 5 animals had died and 3 out of the 5 remaining animals showed signs of anaphylaxis, none of the 10 animals receiving the same dose of PEG-SOD showed any signs of anaphylaxis.

When a PEG-SOD adduct containing approximately 6 strands of 5000 MW PEG per molecule of SOD was tested using the same protocol, by the fifth challenge, two out of 10 animals died and 4 of the remaining animals showed signs of anaphylaxis. Therefore, the PEG-SOD produced by the present invention, containing 3 strands of 72,000 MW PEG per SOD was less immunogenic than a PEG-SOD containing twice as many strands of 5,000 MW PEG.

EXAMPLE 2

Following the method of Example 1, PEGs of high molecular weight were coupled to bovine Cu, Zn SOD. Products with 5 strands of 100,000 MW PEG and 3 strands of 120,000 MW PEG were found to be less immunogenic in mice than native SOD or a product with 4 strands of 35,000 MW PEG.

EXAMPLE 3

The serum persistence of native SOD was compared to that of PEG-SODs prepared by the method of Example 1 using adult female Swiss Webster mice. 100 micrograms of SOD was injected intravenously. Blood was collected at regular intervals and the plasma was assayed for specific PEG-SOD activity by an electrophoretic method that separates PEG-SOD from mouse SOD. One PEG-SOD adduct tested contained 2 strands of about 65,000 MW PEG and another contained 4 strands of about 40,000 MW PEG. The half-life for the disappearance of native SOD in mice was 5-10 minutes, while the half-life for the disappearance of both of these PEG-SOD adducts was greater than 36 hours and PEG-SOD activity

could be detected in the blood of these animals for at least 9 days. Another preparation containing an average of 2.6 strands of about 45,000 MW also produced a half-life exceeding 36 hours.

5

EXAMPLE 4

An aqueous solution of PEG (Union Carbide) which was labelled as 100,000 MW (weight average determined by intrinsic viscosity), but with an average MW of about 50,000 measured by HPLC, was size-fractionated by
10 ultrafiltration using a Millipore Minitan apparatus equipped with a 300,000 (protein standard) molecular weight cut-off membrane. The size-fractionated product was dried under vacuum. Analysis by HPLC showed that the average MW of the sample increased from 50,000 to 100,000 after
15 ultrafiltration. To a solution containing 3.77 grams of such size-fractionated PEG in 100 ml of dry acetonitrile, 1.39 grams of cyanuric chloride in 2.8 ml of dry acetonitrile was added. After standing for 3 days at 24°C the solution was diluted with an equal volume of
20 acetonitrile and then clarified by filtration. The solvent was removed by evaporation under vacuum at 30°C and the residue was redissolved in 120 ml of dry toluene. The product was precipitated by the addition of 360 ml of dry hexane. The product was reprecipitated once more from
25 toluene using petroleum ether and dried under vacuum to yield cyanuric chloride-activated PEG. Size-exclusion HPLC demonstrated that the MW of the PEG did not change as a result of activation. Varying amounts of activated PEG, obtained as described above, were tested for ability to
30 couple to bovine Cu, Zn SOD, present at a constant level of 1 mg/ml. The final PEG concentrations ranged from 5 to 100 mg/ml. After reacting for 24 hours at 24°C, the mixtures were assayed by HPLC with UV detection for the formation of PEG-SOD and also for the amount of residual SOD.

Table 1

	PEG:SOD		Percent Conversion of SOD To Adduct	Adduct Peak MW*
	(w:w)	(M:M)		
5	5	1.7	25%	-
	10	3.3	29%	90K
	20	6.6	62%	140K
10	50	16.7	90%	150K
	75	24.0	95%	200K
	100	33.3	100%	200K

* Determined by HPLC on TSK PW columns calibrated with commercial PEG standards.

As shown in Table 1, at a 50:1 (w/w), PEG to SOD ratio (16.6:1 molar ratio), 90% of the SOD was converted to PEG-SOD with an average molecular weight of 150,000. At greater PEG to SOD ratios, both the amount of SOD converted to PEG-SOD and the molecular weight of the adduct increased. The resulting sizes of the adducts indicate that up to two strands of 100,000 MW PEG can be attached to SOD under these conditions using cyanuric acid as the coupling agent.

The serum persistence in mice was measured for the PEG-SOD products in the 10:1 (w:w) and 75:1 (w:w) PEG to SOD reactions listed in Table 1. The same methods used in Example 3 above, were employed. Half-lives of at least 36 hours were obtained for both products tested.

EXAMPLE 5

Ten grams of 100 kilodalton polyethylene glycol (100K PEG; Union Carbide) were freeze-dried for 24 hours to remove any moisture present in the sample. The dried 100K PEG was dissolved in approximately 45 ml of dry acetonitrile. Then 5.12 grams of 1,1-carbonyldiimidazole was added and the reaction mixture was incubated at room temperature for 1.5 hours, and then quenched in deionized water to destroy the excess carbonyl diimidazole.

The pH was maintained at 7 to prevent hydrolysis of the activated PEG. The mixture was then dialyzed for 1 day at 4°C against 4 liters of distilled water using at least 10 changes to remove the acetonitrile and imidazole. After
5 dialysis, the activated PEG was chromatographed on a Sephacryl S-400 column in deionized water in order to separate the activated 100K-PEG from low molecular weight fragments.

To the resulting activated PEG there was added enough SOD
10 to produce a molar ratio of 3 moles of PEG per mole of SOD. 92.8 mg SOD was added to the pool (vol.=108 ml). The mixture was freeze-dried 4 times in order to produce the desired PEG-O-CO-SOD product.

EXAMPLE 6

15 To a solution of 30 grams of polyethylene glycol in 1100 ml of dry dioxane at 35°C, there was slowly added under nitrogen and with stirring 10 grams of sodium hydride. After an additional hour of stirring at 25°C, 15 ml ethyl bromoacetate was added. The solution was stirred at 25°C
20 for 30 minutes, then for two hours at 45°C and the reaction was terminated by addition of 200 ml of water. 400 ml of petroleum ether were then added with stirring. The organic phase was discarded and the viscous, aqueous phase was washed with petroleum ether. The aqueous phase containing
25 the PEG ethyl ester was diluted to approximately 1 L and saponified by raising the temperature to 60-70°C for five hours. Finally, the PEG carboxyl groups were converted to the free acid by acidification to pH 2. The remaining bromoacetic acid was removed by dialysis or gel filtration.
30 The PEG ether thus prepared can be substituted for the succinylated PEG used in Example 1.

Thus, using 9.4 grams of activated polyethylene glycol of a molecular weight of 40,000 and 7.9 mg of bovine SOD there was obtained an adduct containing an average of 3.3 strands

of PEG per molecule of SOD. The molecular weight of the product, as determined by HPLC, was about 140,000. This adduct had greatly reduced immunogenicity in mice.

Using 6.5 grams of polyethylene glycol of a molecular weight of 120,000 and 87 mg of bovine SOD, there was obtained an adduct with a molecular weight of about 245,000 as determined by HPLC.

EXAMPLE 7

The use of the process with human SOD was established.

20 mg samples of activated, succinylated PEG were placed into glass tubes. 100 mcl. of a solution of 3 mg/ml human SOD in 0.2 molar pH 8 phosphate-borate buffer were added. After mixing, 2 mcl samples of each reaction mixture were removed and quenched in 58 mcl of 0.01 M 1:1 sodium acetate:acetic acid buffer in a 250 microliter microtube. To monitor the progress of the reaction, samples were electrophoresed at pH 8.5 using 250 volts for 15 minutes followed by nitroblue tetrazolium staining.

After 3-3.5 hours at room temperature, the solutions were quenched with 45 microliters of 0.03 molar 1:1 acetate buffer per mg PEG. Addition of 0.1 ml of pH 8 reaction buffer and 0.9 ml 30 mM acetate produced a final pH of 6.4. This pH drop and 1:10 dilution stopped further PEG-SOD coupling and hydrolysis. It was found that there was no appreciable further reaction after 30-45 minutes.

While the PEG-SOD with strands of 19,000 Dalton PEG, like free SOD, was no longer detectable in mouse serum one day after injection, the preparation with strands of 30,000 Dalton PEG gave a prolonged persistence time. Human SOD can thus be used for the purposes of this invention.

EXAMPLE 8

A mouse carrageenan-induced paw edema test was employed to estimate anti-inflammatory activity. In the paw edema test 0.03 ml of 1.0% carrageenan in saline was injected

subcutaneously into the right hind foot pads of female Swiss Webster mice and then, 30 minutes later, the animals were dosed subcutaneously with 100 microliters of test compound which was prepared by dilution in saline followed by filter sterilization. Twenty four hours after the carrageenan injection the animals were sacrificed and both hind feet amputated and weighed. Edema was calculated as weight of the injected foot (right) minus weight of un-injected foot (left). The average and standard deviation of the edema weight for each treatment group, including a saline control, was calculated.

At a dose of 30 micrograms per mouse, an adduct with 2.6 strands of 45,000 MW PEG was more effective in reducing the edema than the adduct with 3.4 strands of 21,000 MW PEG (43% inhibition versus 14% inhibition, respectively). An adduct prepared with 3 strands of 120,000 MW PEG demonstrated 41% edema inhibition at a dose of 10 micrograms per mouse. Native, bovine Cu/Zn SOD was inactive in this assay, even when tested at 300 micrograms per mouse, as was an adduct prepared with 14 strands of 5,000 MW PEG when tested at 100 micrograms per animal.

EXAMPLE 9

As an antigenicity assay, a solid-phase, competitive-binding enzyme immunoassay (ELISA) was used to measure the cross-reactivity of PEG-SOD compounds containing bovine Cu,Zn SOD with rabbit antibody directed against highly purified native bovine Cu,Zn SOD. Conjugates of this invention required fewer strands of PEG to reduce antigenicity than compounds prepared with shorter strands, e.g., with 5,000 MW PEG. For example, an adduct with an average of 2.6 strands of 45,000 MW PEG and one with 4 strands of 35,000 MW PEG were one-third and one-tenth as antigenic, respectively, as an adduct with 6 strands of 5,000 MW PEG. The adduct with 4 strands of 35,000 MW PEG

was half as antigenic as the one containing 14 strands of 5,000 MW PEG. Among adducts prepared with less than 4 PEG strands, those with PEG in the 100,000-120,000 MW range were less antigenic than adducts with the same number of
5 shorter strands. Thus, a SOD adduct with 2 PEG strands of MW 120,000 was only one fifth as antigenic as one with 2 strands of 35,000 MW PEG.

WHAT IS CLAIMED IS:

1. A water-soluble substantially non-immunogenic conjugate of a superoxide dismutase coupled with a coupling agent to a polyalkylene glycol which is a polyethylene glycol or polyethylene polypropylene glycol copolymer residue, wherein said glycol has an average molecular weight as determined by HPLC using PEG as standard of about 40,000-1,000,000 and is terminally unsubstituted or substituted by C₁₋₄-alkyl or C₁₋₄-alkoxy groups.
2. A conjugate of Claim 1, wherein the polyalkylene glycol is a polyethylene glycol of average molecular weight of about 40,000-200,000 as determined by HPLC.
3. A conjugate of Claim 2, wherein the polyethylene glycol is a polyethylene glycol of average molecular weight of about 50,000-150,000.
4. A conjugate of Claim 3, wherein 2 to 8 polyethylene glycol groups are coupled to each copper and zinc containing superoxide dismutase molecule.
5. A conjugate of Claim 4, wherein 2 to 6 polyethylene glycol groups are coupled to each superoxide dismutase molecule.
6. A conjugate of Claim 5, wherein the polyethylene glycol is an isopropoxypolyethylene glycol.
7. A conjugate of Claim 1, wherein the terminal group of a polyethylene glycol is attached through a CO link to terminal amino groups of superoxide dismutase lysines.

8. A pharmaceutical composition which contains an amount of a conjugate of Claim 1 effective to produce a therapeutic superoxide dismutase effect.

9. A pharmaceutical composition according to Claim 8
5 which contains an effective anti-inflammatory amount of the conjugate.

10. A pharmaceutical composition according to Claim 8 which contains an amount of the conjugate effective to produce amelioration of oxygen toxicity.

10 11. A pharmaceutical composition according to Claim 8 which contains an amount of the conjugate effective to afford protection against reperfusion injury.

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 88/02530

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : C 12 N 9/96; A 61 K 37/50; A 61 K 37/14																	
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 25%; border-bottom: 1px solid black;">Classification System</td> <td style="border-bottom: 1px solid black;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">IPC ⁴</td> <td style="padding: 5px;">C 12 N; A 61 K</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	IPC ⁴	C 12 N; A 61 K											
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ⁹</th> <th style="width: 70%; border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">EP, A, 0200467 (AJINOMOTO CO. INC.) 5 November 1986 see page 2, lines 30-36; page 3, lines 1-9, 23-36; page 4; claims</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-3, 5-11</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="text-align: center; vertical-align: top; padding: 5px;">--</td> <td style="text-align: center; vertical-align: top; padding: 5px;">4</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">Analytical Biochemistry, volume 131, no. 1, May 1983, Academic Press, (New York, US), C.A. Beauchamp et al.: "A new procedure for the synthesis of polyethylene glycol-protein adducts; effects on function, receptor recognition, and clearance of superoxide simutase, lactoferrin, and γ-macroglobulin", pages 25-33 see pages 27, 29</td> <td style="text-align: center; vertical-align: top; padding: 5px;">4</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">Research Communications in Chemical Pathology and Pharmacology, volume 29, no. 1, July 1980, P.S. Pyatak et al.: "Preparation of a polyethylene glycol: superoxide ./.</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-11</td> </tr> </table>			Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	EP, A, 0200467 (AJINOMOTO CO. INC.) 5 November 1986 see page 2, lines 30-36; page 3, lines 1-9, 23-36; page 4; claims	1-3, 5-11	Y	--	4	Y	Analytical Biochemistry, volume 131, no. 1, May 1983, Academic Press, (New York, US), C.A. Beauchamp et al.: "A new procedure for the synthesis of polyethylene glycol-protein adducts; effects on function, receptor recognition, and clearance of superoxide simutase, lactoferrin, and γ -macroglobulin", pages 25-33 see pages 27, 29	4	X	Research Communications in Chemical Pathology and Pharmacology, volume 29, no. 1, July 1980, P.S. Pyatak et al.: "Preparation of a polyethylene glycol: superoxide ./.	1-11
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="padding: 5px;">25th October 1988</td> <td style="padding: 5px; text-align: right;">15 NOV 1988</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="padding: 5px; text-align: center;">EUROPEAN PATENT OFFICE</td> <td style="padding: 5px; text-align: right;"> P.C.G. VAN DER PUTTEN </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	25th October 1988	15 NOV 1988	International Searching Authority	Signature of Authorized Officer	EUROPEAN PATENT OFFICE	 P.C.G. VAN DER PUTTEN							
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	<p>dismutase adduct, and an examination of its blood circulating life and anti-inflammatory activity", pages 113-127 see the whole article</p> <p>-----</p>	

US 8802530
SA 23940

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0200467	05-11-86	JP-A- 61249388	06-11-86